JACC Vol. 32, No. 2
August 1998; 528–35

Chronic L-Arginine Treatment Increases Cardiac Cyclic Guanosine 5′-Monophosphate in Rats With Aortic Stenosis: Effects on Left Ventricular Mass and Beta-Adrenergic Contractile Reserve

JOZEF BARTUNEK, MD, PHD, STEPHEN DEMPSEY, MD, ELLEN O. WEINBERG, PHD, NOBUHIKO ITO, MD, PHD, MINORI TAJIMA, MD, PHD, SUSANNE ROHRBACH, BA, BEVERLY H. LORELL, MD

Boston, Massachusetts

Objectives. **We tested the hypothesis that nitric oxide (NO) cyclic guanosine 5′-monophosphate (GMP) signaling is deficient in pressure overload hypertrophy due to ascending aortic stenosis, and that long-term L-arginine treatment will increase cardiac cyclic GMP production and modify left ventricular (LV) pressure overload hypertrophy and beta-adrenergic contractile response.**

Background. **Nitric oxide cyclic GMP signaling is postulated to depress vascular growth, but its effects on cardiac hypertrophic growth are controversial.**

Methods. **Forty control rats and 40 rats with aortic stenosis left ventricular hypertrophy ([LVH] group) were randomized to receive either L-arginine (0.40 g/kg/day) or no drug for 6 weeks.**

Results. **The dose of L-arginine did not alter systemic blood pressure. Animals with LVH had similar LV constitutive nitric oxide synthase (cNOS) mRNA and protein levels, and LV cyclic GMP levels as compared with age-matched controls. In rats with LVH L-arginine treatment led to a 35% increase in cNOS protein levels ($p = 0.09$ vs untreated animals with LVH) and a 1.7-fold increase in LV cyclic GMP levels ($p < 0.05$ vs untreated animals with LVH). However, L-arginine treatment did not suppress LVH in the animals with aortic stenosis. In contrast, in vivo LV systolic pressure was depressed in L-arginine treated versus untreated rats with LVH (163 ± 16 vs 198 ± 10 mm Hg, $p < 0.05$). In addition, the contractile response to isoproterenol was blunted in both isolated intact hearts and isolated myocytes from L-arginine treated rats with LVH compared with untreated rats with LVH. This effect was mediated by a blunted increase in peak systolic intracellular calcium in response to beta-adrenergic stimulation.**

Conclusions. **Left ventricular hypertrophy due to chronic mechanical systolic pressure overload is not characterized by a deficiency of LV cNOS and cyclic GMP levels. In rats with aortic stenosis, L-arginine treatment increased cardiac levels of cyclic GMP, but it did not modify cardiac mass in rats with aortic stenosis. However, long-term stimulation of NO-cyclic GMP signaling depressed in vivo LV systolic function in LVH rats and markedly blunted the contractile response to beta-adrenergic stimulation.**

(J Am Coll Cardiol 1998;32:528–35)

Long-term angiotensin-converting enzyme (ACE) inhibition improves left ventricular (LV) function and attenuates progression of LV hypertrophy (LVH) (1–3). Several studies suggest that part of the effects of long-term ACE inhibition are related to an enhanced activation of the nitric oxide (NO)-cyclic guanosine 5′-monophosphate (GMP) pathway (4,5), but the effects of this pathway on cardiac growth are unknown. It has been demonstrated that acute stimulation of the NO-cyclic GMP pathway directly depresses myocardial contractile performance (6–8) and attenuates the contractile response to beta-adrenergic stimulation (8–13). In addition, in vitro studies in vascular smooth muscle cells showed that the NO-cyclic GMP pathway modifies cell growth (14,15).

Recent studies, which agree with this concept, have postulated that deficient NO-cyclic GMP production is a fundamental pathophysiologic mechanism in genetic models of hypertension (16–19), such as the spontaneously hypertensive rat model (16,18) or essential hypertension in humans (19). Matsuoka et al. (18) reported that hypertensive rats are characterized by decreased cardiac cyclic GMP content related to NO production, which could be corrected by long-term L-arginine treatment. They observed that normalization of cardiac cyclic GMP content attenuated progression of cardiac hypertrophy. It is unclear whether these provocative observations are relevant to chronic hypertrophy due to mechanical overload such as aortic stenosis. Accordingly, we tested the hypothesis that LVH in rats with ascending aortic stenosis is characterized by

From the Charles A. Dana Research Institute, the Harvard-Thorndike Laboratory, and the Department of Medicine, Cardiovascular Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts. This study was supported in part by Grant HL-38189 (BHL and EOW) from the National Heart, Lung and Blood Institute, Bethesda, Maryland and by the fellowship award F05 TW05261-01 from the Fogarty International Center, National Institutes of Health, Bethesda, Maryland (JB).

Manuscript received January 14, 1998; revised manuscript received April 17, 1998, accepted April 27, 1998.

Address for correspondence: Dr. Beverly H. Lorell, Cardiovascular Division, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, Massachusetts 02215. e-mail: blorell@bidmc.harvard.edu.

0735-1097/98/$19.00
S0735-1097(98)00262-9

Abbreviations and Acronyms

ACE	=	angiotensin-converting enzyme
BW	=	body weight
$[Ca^{2+}]_i$	=	intracellular calcium
GMP	=	guanosine 5′-monophosphate
LV	=	left ventricular
LV devP	=	left ventricular developed pressure
LV dP/dt	=	first derivative of left ventricular pressure
LVEDP	=	left ventricular end-diastolic pressure
LVH	=	left ventricular hypertrophy
LVSP	=	left ventricular systolic pressure
NO	=	nitric oxide
NOS	=	nitric oxide synthase

a deficiency in cardiac cyclic GMP production. Second, we predicted that L-arginine treatment would increase NO-mediated cyclic GMP generation and directly suppress hypertrophic growth in vivo. Third, we postulated that augmented NO-cyclic GMP signaling by long-term L-arginine will modify both in vivo LV systolic performance and the contractile response to beta-adrenergic stimulation.

Methods

L-arginine treatment protocol. Weanling male Wistar rats (3 to 4 weeks old, 75 to 100 g, Charles River Breeding Laboratories, Wilmington, Delaware) were banded by placing a stainless steel clip of 0.6-mm internal diameter on the ascending aorta via a thoracic incision as previously described (2,21). Age-matched animals served as a control group. Rats were fed normal rat chow and water ad libitum.

One week after surgery, rats with aortic stenosis and age-matched controls were randomized to receive either L-arginine in drinking water (0.40 g/kg/day) or no treatment. The dose of L-arginine was chosen on the basis of prior studies in the literature demonstrating that the dose is sufficient to increase cardiac cyclic GMP content (18,20), and on the basis of a pilot-dosing experiment, in which this dose did not affect systemic tail-cuff blood pressure in control animals (n = 6) over a 1-week period. There were four groups in the study: L-arginine treated rats with aortic stenosis (n = 14), untreated rats with aortic stenosis (n = 14) and age-matched controls receiving L-arginine treatment (n = 20) and not receiving L-arginine (n = 20). This randomized treatment continued for 6 weeks.

In vivo measurements. Animals were monitored daily. Body weight was measured weekly. In vivo tail-cuff systemic blood pressure was measured during weeks 4 to 6 of treatment. At the end of the treatment period, in vivo LV pressure measurements were performed before sacrifice as previously described (2). After the animals were sacrificed, tibia, lungs and kidneys were stored at −80°C for further analysis. Tibial length was used as an index of body growth independent of body mass.

Effect of isoproterenol in isolated perfused hearts. Isolated hearts were evaluated using the isovolumic buffer-perfused rat heart preparation with constant coronary flow as described elsewhere (2). All hearts were allowed to stabilize for 15 minutes. Left ventricular diastolic pressure–volume curves were generated as previously described (2). Then, hearts from each group were perfused with isoproterenol at doses of 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} mol/L, each for 5 min. At the end of the experiment, hearts were removed and dissected within 15 s, and the left and right ventricles were quickly weighed and frozen in liquid nitrogen for further analysis.

Effects of isoproterenol in isolated myocytes. At the end of treatment period, additional rats (two to three per group) were used for isolated myocyte experiments. Left ventricular myocytes were prepared as previously described (21,22). Intracellular calcium $[Ca^{2+}]_i$ was measured with the Ca^{2+}-sensitive fluorescence indicator indol-AM (Molecular Probes Inc, Eugene, Oregon) as described elsewhere (21,22). To investigate the response to beta-adrenergic stimulation after chronic L-arginine treatment, isolated myocytes were superfused with oxygenated HEPES-buffered solution (pH = 7.40, 37°C) and paced at 0.5 Hz. After recording baseline data, the cells were superfused with isoproterenol at concentrations of 10^{-10}, 10^{-9} and 10^{-8} mol/L. Simultaneous measurements of cell shortening and $[Ca^{2+}]_i$ were measured after 5 min of superfusion at each concentration.

Measurements of tissue cyclic GMP levels. The frozen tissues from the left ventricle and kidney were rapidly weighed and pulverized under liquid nitrogen. A part of the frozen powder was taken to quantitate the ratio of frozen to dry weight. The remaining powder was homogenized in cold 6% trichloracetic acid to obtain a final 10% homogenate. The homogenate was centrifuged for 20 min at 10,000 rpm and the supernatant was recovered and washed 4 times with 5 volumes of water-saturated ethyl ether. Final aqueous extract was dried at 60°C under a stream of nitrogen. Left ventricular cyclic GMP levels were determined by enzymatic assay (Amersham, Life Science, UK) and expressed as picomolar per gram dry weight.

Ribonuclease protection assay of nitric oxide synthase mRNA. Total RNA was isolated from frozen LV tissue by the guanidinium/cesium chloride method. Left ventricular tissue was analyzed from four untreated control and rats with aortic stenosis. Labeled riboprobes were generated using MAXIcript In Vitro Transcription kit (Ambion, Austin, Texas) and ^{32}P-alpha-UTP. Labeled RNA was separated from unincorporated nucleotides by spin chromatography (Chromaspin-100, Clontech, Palo Alto, California). The constitutive nitric oxide synthase (cNOS) probe was linearized with *XhoI* and yielded a 303 base-pair (bp) fragment (7). The rat beta-actin probe was derived from clone pSKrBac and yielded a 150 bp fragment after linearization with *XhoI*. Fifty micrograms of total RNA was hybridized to 1×10^5 cpm of cNOS RNA and 1×10^4 cpm of beta-actin cRNA and then treated with RNase A/RNase T_1 (Ambion). After RNase inactivation and precipitation of the protected fragments, the samples were separated on a 5%

Table 1. Left Ventricular Hypertrophy and Blood Pressure

Group	Body Wt (g)	Tibial Lt (mm)	LV Wt (g)	LV Wt/Body Wt (g/kg)	BP (mm Hg)
C (n = 10)	400.8 ± 5.8	39.2 ± .4	.99 ± .04	2.53 ± .08	126 ± 1
C-L-arg (n = 10)	386.5 ± 11.6	39.3 ± .4	1.01 ± .02	2.56 ± .09	132 ± 5
LVH (n = 10)	391.4 ± 12.1	39.9 ± .3	1.61 ± .05*	4.01 ± .16*	109 ± 6*
LVH-L-arg (n = 11)	407.8 ± 7.1	40.2 ± .4	1.70 ± .03*	4.02 ± .09*	110 ± 5*

*$p < 0.05$ versus controls.

Body Wt = body weight; BP = systolic tail-cuff blood pressure; C = control untreated animals; C-L-arg = L-arginine treated control animals; LV Wt = left ventricular weight; LVH = untreated aortic banded animals; LVH-L-arg = L-arginine treated animals with aortic stenosis; Tibial Lt = tibial length.

polyacrylamide gel. The gel was exposed to autoradiographic film (Kodak MR) overnight, and hybridization signals were quantified with Image Quant Software (Molecular Dynamics, Sunnyvale, California). Constitutive nitric oxide synthase levels were normalized to beta-actin mRNA levels.

Western blot analysis of nitric oxide synthase proteins. Left ventricular tissue was rapidly homogenized in 5 volumes of a lysis buffer containing 10 mmol/L Tris HCl, 1 mmol/L orthodium vanadate and 1% SDS. Left ventricular tissue was analyzed from 11 untreated control rats, 9 L-arginine treated control rats and 15 L-arginine treated and untreated rats with aortic stenosis. Homogenized samples were microwaved for 15 s and centrifuged for 20 min at 10,000 rpm. Supernatant was recovered and proteins were quantified using Lowry assay (Sigma Chemical, St. Louis, Missouri). One hundred micrograms of denaturated protein per lane were loaded and separated on a 10% SDS-PAGE gel. Random samples from each group were loaded into each gel. Eight microliters of human aortic endothelial cell proteins (concentration 1 mg/ml) were loaded into each gel and served as a positive control. Separated proteins were transferred to a nitrocellulose membrane. Membranes were exposed to constitutive or inducible NOS specific monoclonal antibody (both at 1:800, Transduction Laboratories, Lexington, Kentucky) for 1 h followed by six washes and incubation with a goat antimouse secondary antibody coupled to peroxidase activity (1:5,000). The membranes were incubated with chemiluminiscence detection reagent (Amersham, Life Science) and exposed to Kodak film for 30 to 40 s.

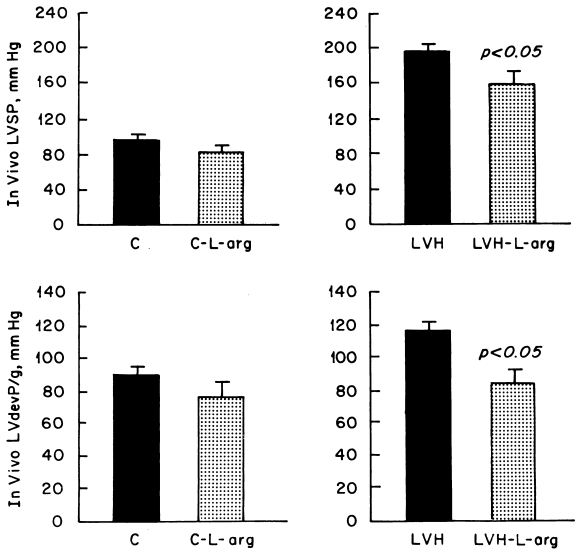
Statistical analysis. All data are expressed as mean ± SEM. Student unpaired *t* test was used where appropriate. Comparison between groups was performed by analysis of variance (ANOVA) comparison or ANOVA for repeated measures, where appropriate, followed by Fisher's exact test for post hoc analyses. A p value <0.05 was considered significant.

Results

Effect of L-arginine on left ventricular hypertrophy. In both the control rats and the rats with aortic stenosis, L-arginine had no effect on body weight and on tibial length, an index of growth independent from body fat, muscle or fluid homeostasis among the groups (Table 1). As shown in Table 1, systolic tail-cuff blood pressure was slightly lower in untreated rats with aortic stenosis compared with control rats. At the dose used in this long-term trial, L-arginine had no effect on systolic blood pressure in control rats or rats with aortic stenosis. The effect of L-arginine on LV growth is shown in Table 1. Left ventricular weight and LV/body weight ratio were significantly greater in rats with aortic stenosis than in control rats. However, LV weight was similar between L-arginine treated rats and untreated rats with aortic stenosis.

In vivo left ventricular function. To assess the effect of L-arginine on in vivo LV function, LV pressure was measured in closed-chest animals before sacrifice (Figure 1). In control rats, absolute LV systolic pressure (LVSP) and LV developed pressure/g LV (LVdevP/g) were similar in L-arginine treated and untreated rats (n = 10 per group). In untreated rats with

Figure 1. Bar graph showing in vivo LVSP measurements. **Upper left,** in vivo LVSP in control animals. **Upper right,** in vivo LVSP in rats with aortic stenosis. **Lower left,** in vivo LV developed pressure/g (LVdevP/g) in control rats. **Lower right,** In vivo LVdevP/g in rats with aortic stenosis. C = control rats not receiving drugs; C-L-arg = control rats treated with L-arginine; LVH = rats with aortic stenosis not receiving drugs; LVH-L-arg: rats with aortic stenosis treated with L-arginine.



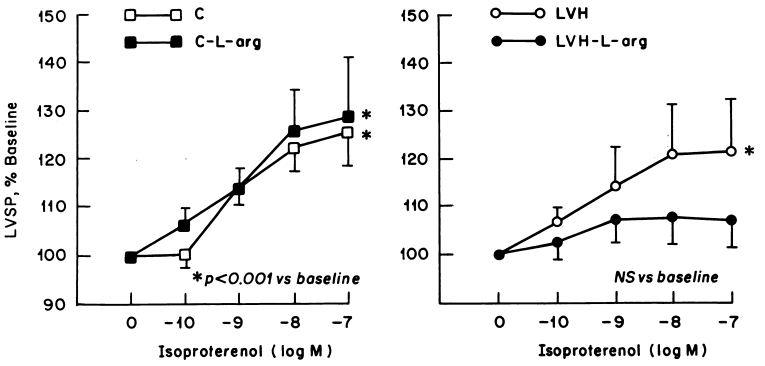


Figure 2. Graph showing the contractile response to isoproterenol in isolated intact hearts from control animals **(left panel)** and rats with aortic stenosis **(right panel).** Abbreviations as in Figure 1.

aortic stenosis, in vivo LVSP and LVdevP/g were higher than in control animals. However, both absolute LVSP and LVdevP/g were significantly lower in L-arginine treated rats (n = 11) compared with untreated rats with aortic stenosis (n = 13). In addition, maximum and minimum first derivative of LV pressure (LV dP/dt) tended to be lower in L-arginine treated than in untreated rats with aortic stenosis (6,625 ± 1,080 vs 8,196 ± 438 mm Hg/s, p = 0.13, and 5,857 ± 964 vs 7,156 ± 478 mm Hg/s, p = 0.19, respectively).

In vivo LV end-diastolic pressure (LVEDP) was higher in rats with aortic stenosis than in control rats (12.9 ± 2.6 vs 6.5 ± 1.0 mm Hg, $p < 0.01$). However, LVEDP was similar in animals with aortic stenosis with and without L-arginine (14.8 ± 1.8 vs 12.9 ± 2.6 mm Hg; p = NS).

Contractile response to isoproterenol in isolated hearts. Isolated heart studies were used to examine the contractile response to beta-adrenergic stimulation in L-arginine treated and untreated rats. Hearts in all groups were perfused at a comparable flow rate per gram (≈18 ml/min/g) and at a similar heart rate (≈280 beats/min). As expected, there was an upward and leftward shift of the LV diastolic pressure–volume relation in rats with aortic stenosis compared with control rats. However, the LV diastolic pressure–volume relation showed no difference in LV diastolic chamber distensibility in the presence or absence of L-arginine treatment (data not shown). Then, LV volume was adjusted to achieve a comparable level of LVEDP in all groups (11 mm Hg). At this level of LVEDP, LV volume was similar between treated and untreated control rats (0.22 ± 0.04 vs 0.19 ± 0.02 ml) and rats with aortic stenosis (0.14 ± 0.02 vs 0.13 ± 0.02 ml). Under these perfusion conditions, LV systolic pressure was similar in L-arginine treated and untreated control rats (124 ± 10 vs 110 ± 5 mm Hg), and in L-arginine treated and untreated rats with aortic stenosis (161 ± 13 vs 151 ± 14 mm Hg). The dose response of coronary perfusion pressure and heart rate to isoproterenol was similar between treated and untreated control rats and rats with aortic stenosis (data not shown). There was no significant change in LVEDP during isoproterenol infusion in any group. As shown in Figure 2, in control animals (left panel), isoproterenol caused a similar dose-dependent increase in LV systolic pressure in hearts from both L-arginine treated and untreated animals ($p < 0.001$ vs baseline). Hearts from untreated rats with aortic stenosis also demonstrated a significant dose-dependent increase in LVSP (Fig. 2, right panel, $p < 0.001$ vs baseline), whereas there was no increase in LVSP in hearts from L-arginine treated rats with aortic stenosis (p = NS).

Response to isoproterenol in isolated myocytes: effect on contractility and $[Ca^{2+}]_i$. We then examined effects of isoproterenol on contractility of isolated myocytes. The baseline function of LV myocytes from control and hypertrophied hearts is shown in Table 2. Figure 3 shows the effects of isoproterenol on myocyte shortening. Isoproterenol induced a similar, dose-dependent increase in fractional shortening in myocytes from treated and untreated control animals ($p < 0.001$ vs baseline; Fig. 3, left panel). In hypertrophied myocytes from animals with aortic stenosis, the relative increase in fractional shortening (Fig. 3, right panel) was much higher in hypertrophied myocytes from untreated rats with aortic stenosis than in myocytes from L-arginine treated rats with aortic stenosis ($p < 0.05$ by ANOVA).

The dose-dependent effects of isoproterenol on systolic $[Ca^{2+}]_i$ are shown in Figure 4. Isoproterenol induced a similar increase in systolic $[Ca^{2+}]_i$ in control myocytes from L-arginine treated and untreated rats (Fig. 4, left panel). In hypertrophied myocytes, the relative increase in systolic $[Ca^{2+}]_i$ tended to be lower in myocytes from L-arginine treated than in untreated rats with aortic stenosis (p = 0.08 by ANOVA; Fig. 4, right

Table 2. Baseline Characteristics of Isolated Myocytes

	C (n = 9)	C-L-ARG (N = 8)	LVH (n = 9)	LVH-L-arg (n = 8)
Diastolic length (μm)	122.6 ± 4.1	125.1 ± 4.5	151.1 ± 5.1*	155.3 ± 4.2*
Systolic shortening (μm)	10.9 ± 1.5	11.1 ± 1.1	13.7 ± 2.6	14.9 ± 2.5
Fractional shortening (%)	8.7 ± 1.1	9.1 ± .8	8.8 ± .8	9.7 ± 1.5
End-diastolic $[Ca^{2+}]_i$ (nM)	79 ± 13	76 ± 14	78 ± 17	99 ± +17
Peak-systolic $[Ca^{2+}]_i$ (nM)	534 ± 137	542 ± 78	588 ± 79	598 ± 54

*$p < 0.05$ versus controls. Abbreviations as in Table 1.

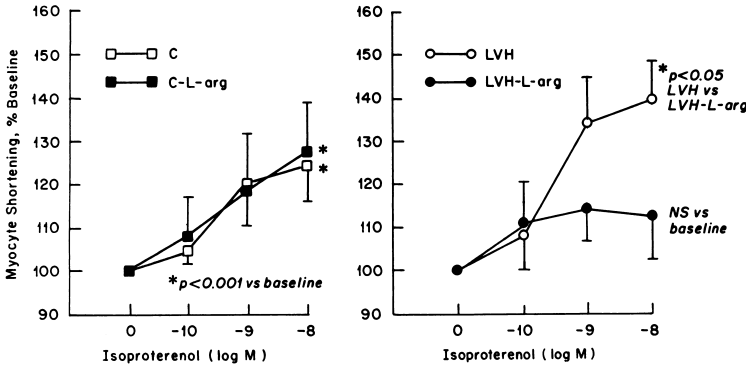


Figure 3. Graph showing the contractile response to isoproterenol in isolated control **(left panel)** and hypertrophied **(right panel)** myocytes. Abbreviations as in Figure 1.

panel). There was no change in diastolic $[Ca^{2+}]_i$ in any group (data not shown).

Tissue cyclic GMP levels. To determine whether cardiac cyclic GMP levels were intrinsically depressed in the experimental model of pressure overload, we measured tissue LV cyclic GMP levels in all groups (Table 3). Cyclic GMP levels were similar in LV tissues of rats with aortic stenosis and age-matched control rats. Long-term administration of L-arginine significantly elevated LV cyclic GMP levels in rats with aortic stenosis compared with untreated rats with aortic stenosis. As a positive control of the effects of long-term L-arginine treatment on NO-mediated signaling, cyclic GMP content was also assessed in kidneys. Renal cyclic GMP levels were higher in untreated animals with aortic stenosis compared with untreated control animals. Furthermore, L-arginine treatment significantly increased renal cyclic GMP content in control rats as well as in rats with aortic stenosis.

Left ventricular nitric oxide synthase mRNA and protein levels. Levels of LV cNOS mRNA, which were measured by RNase protection assay and expressed as densitometric units normalized to beta-actin, were similar between untreated control animals and animals with aortic stenosis (0.181 ± 0.021 vs 0.152 ± 0.017 U, p = NS).

Steady-state levels of LV cNOS were determined by Western analysis. On each gel, samples from each group were normalized to the signal obtained from the control human endothelial cell protein standard. Left ventricular cNOS protein levels were similar between untreated control rats and animals with aortic stenosis (0.811 ± 0.084 vs 0.703 ± 0.052 U, p = NS). In rats with aortic stenosis, L-arginine treatment induced a 35% increase in cNOS protein levels compared with untreated animals (p = 0.09). In control animals, L-arginine induced a 25% increase in LV cNOS protein levels (p = 0.11). LV inducible NOS protein levels were not detectable in any group.

Discussion

Previous studies in genetic models of essential hypertension (16–19) have raised the provocative hypothesis that a fundamental defect in pressure overload hypertrophy is deficient NO-cyclic GMP signaling, and have shown that long-term L-arginine administration is associated with an increase of cardiac cyclic GMP levels and regression of cardiac hypertrophy (20). However, it is not known whether NO-cyclic GMP signaling is deficient in other models of pressure overload and whether long-term L-arginine treatment can modify hypertrophic growth and/or myocardial performance in compensatory experimental mechanical pressure overload hypertrophy. Thus, the present study investigated the effect of long-term L-arginine treatment on cardiac hypertrophy and myocardial function in rats with compensatory pressure overload hypertrophy due to ascending aortic stenosis. In contrast with the genetic models of the spontaneously hypertensive rat, this

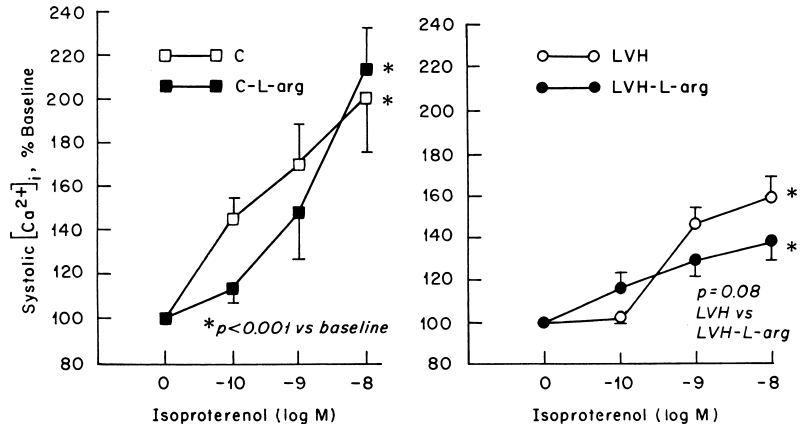


Figure 4. Graph showing the relation between isoproterenol infusion and changes in peak systolic intracellular calcium in control **(left panel)** and hypertrophied **(right panel)** myocytes. Abbreviations as in Figure 1.

Table 3. Tissue Cyclic GMP Levels

	C	C-L-arg	LVH	LVH-L-arg
LV cyclic GMP (pmol/g dry weight)	27.71 ± 4.81	37.22 ± 4.86	36.09 ± 4.39	56.53 ± 7.52*†
Renal cyclic GMP (pmol/g dry weight)	19.83 ± 5.45	89.05 ± 16.96†	43.31 ± 8.94	106.95 ± 21.14*†

n = 5 to 12 per group. *$p < 0.05$ versus control animals; †$p < 0.05$ versus untreated animals. Abbreviations as in Table 1.

model provides a pure mechanical pressure overload on the heart in the absence of systemic hypertension (2,23), and separates potential confounding effects of genetic alterations in NO-cyclic GMP signaling.

Nitric oxide-cyclic GMP signaling in compensatory hypertrophy. Prior studies (1,2,24) have demonstrated that long-term ACE inhibition promotes regression of pressure overload hypertrophy. Because long-term ACE inhibition may lead to an enhanced action of cardiac NO-cyclic GMP pathway via inhibition of bradykinin degradation (4,5), this has led to speculation that cardiac NO-cyclic GMP signaling may be altered in pressure overload hypertrophy.

Our data show that compensatory pressure overload hypertrophy is not associated with a deficiency in LV cyclic GMP production. This finding contrasts with data obtained in a genetic spontaneously hypertensive rat model (18); however, it corroborates previous studies (25,26) that reported an increase rather than deficit in cardiac cyclic GMP content in experimental compensatory pressure overload hypertrophy.

Our data also show that both LV mRNA and LV protein levels of cNOS are similar between untreated control rats and rats with aortic stenosis. In contrast, studies in spontaneously hypertensive rats reported an upregulated cNOS activity in cardiac endothelial cells (27) as well as in isolated myocytes (28,29). Although it is unclear what underlies these discrepancies, it is possible that different mechanisms of increased systolic load may have distinct effects on each component of the L-arginine/NO signaling cascade, including interplay with other mediators such as atrial natriuretic peptide, endothelin, prostaglandins or neuregulins. Taken together, the current data suggest that the transcriptional and functional response of the L-arginine/NO pathway to pressure overload is complex and not predictable purely from an increase in systolic load per se.

Effects of L-arginine/nitric oxide-cyclic GMP pathway on cardiac hypertrophy. Several studies have shown a growth suppressant effect of NO and its donors using in vitro preparations of vascular smooth muscle cells (14,15) mediated by cyclic GMP dependent kinases (30). However, it is not yet known whether NO unambiguously suppresses the growth of cardiac myocytes in culture or in intact adult hearts. Harding et al. (31) reported that a high dose of nitroglycerin reduced basal and phenylephrine-induced protein synthesis in cultured neonatal myocytes. It is possible that at nonphysiologic high doses of NO donors in vitro in the absence of hemoglobin, generation of peroxynitrate, rather than direct effect of NO, may be responsible for growth inhibition in cultured cells (32). Matsuoka et al. (18) demonstrated that long-term L-arginine administration attenuated cardiac mass in the genetic spontaneously hypertensive rat model. In that study, cardiac cyclic GMP levels were markedly depressed in the spontaneously hypertensive rats and L-arginine administration normalized depressed cyclic GMP production without changes in mean arterial pressure. Thus, a higher availability of L-arginine as a substrate for an upregulated NOS (27–29) might be beneficial in suppressing myocyte growth in spontaneously hypertensive rats.

In the present study, the dose of L-arginine was sufficient to achieve the metabolic end point of an increase in renal (33) and LV cyclic GMP (18). It is notable that although L-arginine treatment increased LV cyclic GMP in the rats with aortic stenosis, the extent of LV mass remained unchanged. Thus, in the presence of persistent severe LVSP overload due to aortic stenosis, LV cyclic GMP levels are not decreased and further augmentation of NO-mediated cyclic GMP levels does not suppress cardiac growth. The different outcome of long-term L-arginine treatment on hypertrophic growth in the mechanical pressure overload, in contrast to the genetic models of hypertension such as a spontaneously hypertensive rat, suggests that L-arginine stimulation of NO-cyclic GMP does not change LV mass when basal LV cyclic GMP levels are preserved. However, neither our nor previous studies address the possibility that L-arginine treatment might have modified in vivo LV concentric remodeling (wall thickness/chamber diameter ratio), which has not been assessed, without changes in the extent of LV mass.

Effects of L-arginine/NO pathway on myocardial performance in LV hypertrophy. The present study demonstrates for the first time that long-term stimulation of NO-mediated cyclic GMP depresses LV contractile function. Because L-arginine treatment did not alter tail-cuff systolic blood pressure, the reduction in LVSP generation in vivo in rats with aortic stenosis can be attributed to a direct negative inotropic effect on myocardium. This effect is concordant with acute modulation of contractile performance by NO in the absence of beta-adrenergic stimulation (6–8,34,35) which is attributed predominantly to a myofilament desensitization (8,21,36,37). We recently demonstrated that myofilament desenzitization by NO donors and 8-bromo-cyclic GMP is likely due to altered Na^+-H^+ exchange resulting in a reduction of intracellular pH (21). Of note, unchanged tail-cuff pressure and reduced LV pump function in the presence of a fixed aortic stenosis in the L-arginine treated rats with aortic stenosis suggests a reflexive

adjustment of systemic resistance to maintain systemic blood pressure.

In this study, we also observed that long-term L-arginine treatment caused a blunted beta-adrenergic contractile response to isoproterenol in hypertrophied hearts and myocytes. Several studies have shown the acute depression of the contractile response to beta-adrenergic stimulation by endogenous NO in cytokine-treated myocytes (9), in isolated papillary muscles (11) and in human studies (10,12). An attenuated contractile response to isoproterenol mediated by NO was also observed in failing myocytes due to pacing-induced heart failure (13). Our observation that long-term L-arginine treatment depresses the beta-adrenergic inotropic response in compensatory hypertrophy is novel, and suggests an intervention with the potential to limit pathologic beta-adrenergic stimulation in the presence of LVH.

The present study suggests that in the presence of beta-adrenergic stimulation, the reduction of the force generation by endogenous NO-mediated cyclic GMP signaling is mediated by a blunted increase in peak systolic calcium in response to beta-adrenergic stimulation. This idea is supported by prior studies demonstrating the NO-cyclic GMP mediated suppression of the inward L-type calcium current in stimulated myocytes (7,38). This effect may also be related to the interplay between the NO-cyclic GMP pathway and the regulatory inhibitory G protein, which has been recently described by Hare et al (39). It should be noted that although beta-adrenergic receptor density was not measured in our study, the beta-adrenergic contractile reserve was intact in myocytes from untreated rats with aortic stenosis at this stage of compensatory hypertrophy. Hence, the impaired response to isoproterenol is consistent with elevated levels of LV cyclic GMP in L-arginine treated animals with LVH. Thus, our study and previous studies (7,8,21,36,37) support the hypothesis of at least two complementary mechanisms involved in NO-cyclic GMP mediated modulation of myocardial contractility. First, myofilament desensitization appears to underlie the negative inotropic effects in the absence of beta-adrenergic stimulation, and second, inhibition of adrenergically stimulated calcium signaling appears to modulate the inotropic response in the presence of beta-adrenergic stimulation.

Study limitations. There are several limitations of the current study. First, we investigated the effects of L-arginine treatment at the stage of early compensated LVH. Thus, it remains uncertain whether L-arginine treatment will augment cyclic GMP signaling at a later stage of decompensated pressure overload hypertrophy. Second, we did not directly measure steady-state or stimulated endogenous cardiac NO production. Accordingly, it cannot be ruled out that some of the effects of L-arginine treatment on cardiac cyclic GMP are not related to NO. Third, it is unclear why L-arginine caused a greater increase in LV cyclic GMP levels in animals with aortic stenosis than in control animals. We speculate that this could be related to differences in L-arginine transport in control and hypertrophied hearts (40).

Study implications. In the present study, L-arginine treatment elevated LV levels of cyclic GMP in rats with aortic stenosis. The augmentation of cyclic GMP signaling failed to change LV mass in these rats. Nonetheless, long-term L-arginine treatment caused a mild depression of in vivo LV contractile function in rats with aortic stenosis and blunted the beta-adrenergic contractile response to isoproterenol in intact hypertrophied hearts and isolated myocytes. Beneficial or adverse long-term effects of the stimulation of NO-cyclic GMP signaling and interference with beta-adrenergic signaling on myocardial function, myocardial energetics, and the later transition to cardiac failure in the presence of mechanical pressure overload are unknown and require further investigation.

We greatly appreciate the thoughtful comments and suggestions of Drs. Thomas W. Smith, Ralph A. Kelly, Cardiovascular Division, Brigham and Women's Hospital, Boston, Massachusetts, and Thomas H. Hintze, Department of Physiology, New York Medical College, Valhalla, New York, to our study.

References

1. Nakashima Y. Fouad FM, Tarazi RC. Regression of left ventricular hypertrophy from systemic hypertension by enalapril. Am J Cardiol 1984;53: 1044–9.
2. Weinberg EO, Schoen FJ, George D, et al. Angiotensin-converting enzyme inhibition prolongs survival and modifies the transition to heart failure in rats with pressure overload hypertrophy due to ascending aortic stenosis. Circulation 1994;90:1410–22.
3. Friedrich SP, Lorell BH, Rousseau MF, et al. Intracardiac angiotensin-converting enzyme inhibition improves diastolic function in patients with left ventricular hypertrophy due to aortic stenosis. Circulation 1994;90:2761–71.
4. Anning PB, Grocott-Mason RM, Lewis MJ, Shah AM. Enhancement of left ventricular relaxation in the isolated heart by an angiotensin-converting enzyme inhibitor. Circulation 1995;92:2660–5.
5. Wiemer G, Scholkens BA, Bewcker RH, Busse R. Ramiprilat enhances endothelial autocoid formation by inhibiting breakdown of endothelium-derived bradykinin. Hypertension 1991;18:558–63.
6. Paulus WJ, Vantrimpont PJ, Shah AM. Paracrine coronary endothelial control of left ventricular function in humans. Circulation 1995;92:2119–26.
7. Balligand JL. Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle function by an endogenous nitric oxide signaling system. Proc Natl Acad Sci USA 1993;90:347–51.
8. Kelly RA, Balligand JL, Smith TW. Nitric oxide and cardiac function. Circ Res 1996;79:363–80.
9. Ungureanu-Longrois D, Balligand J-L., Okada I, et al. Contractile responsiveness of ventricular myocytes to isoproterenol is regulated by induction of nitric oxide synthase activity in cardiac microvascular endothelial cells in heterotypic primary culture. Circ Res 1995;77:486–93.
10. Hare JM, Loh E, Creager MA, Colucci WS. Nitric oxide inhibits the positive inotropic response to β-adrenergic stimulation in humans with left ventricular dysfunction. Circulation 1995;92:2198–203.
11. Mohan P, Brutsaert DL, Paulus WJ, Sys SU. Myocardial contractile response to nitric oxide and cGMP. Circulation 1996;93:1223–9.
12. Bartunek J, Shah AM, Vanderheyden M, Paulus WJ. Dobutamine enhances cardiodepressant effects of receptor-mediated coronary endothelial stimulation. Circulation 1997;95:90–6.
13. Yamamoto S, Tsutsui H, Tagawa H, et al. Role of myocyte nitric oxide in β-adrenergic hyporesponsiveness in heart failure. Circulation 1997;95: 1111–4.
14. Carg UC, Hassid A. Nitric-oxide vasodilators and 8 bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 1989;83:1774–7.
15. Dubey RK, Jackson EK, Luscher TF. Nitric oxide inhibits angiotensin

II-induced migration of rat aortic smooth muscle cell. J Clin Invest 1995;96: 141–9.

16. Kuo JF, Davis CW, Tse J. Depressed cardiac cyclic GMP-dependent protein kinase in spontaneously hypertensive rats and its further depression by guanethidine. Nature 1976;261:335–6.
17. Hasegawa T, Takagi S, Nishimaki K, Morita K, Nakajima S. Impairment of L-arginine metabolism in spontaneously hypertensive rats. Biochem Int 1992;26:653–8.
18. Matsuoka H, Nakat M, Keisuke K, Yoshinori K, Nomura G, Toshima H, Imaizumi T. Chronic L-arginine administration attenuates cardiac hypertrophy in spontaneously hypertensive rats. Hypertension 1996;27:14–8.
19. Forte P, Copland M, Smith LM, Milne E, Sutherland J, Benjamin N. Basal nitric oxide synthesis in essential hypertension. Lancet 1997;349:837–42.
20. Mitani, Y. Maruyama, K, Sakurai, M. Prolonged administration of L-arginine amelioartes chronic pulmonary hypertension and pulmonary vascular remodeling in rats. Circulation 1997;96:689–97.
21. Ito N, Bartunek J, Spitzer KW, Lorell BH. Effects of the nitric oxide donor sodium nitroprusside on intracellular pH and contraction in hypertrophied myocytes. Circulation 1997;95:2303–11.
22. Ikenouchi H, Barry WH, Bridge JHB, Weinberg EO, Apstein CS, Lorell BH. Effects of angiotensin II on contractility, intracellular Ca^{2+}, I_{ca} and pH: studies in isolated beating hearts and myocytes loaded with the indicator Indo 1. J Physiol (London) 1994;480:203–15.
23. Litwin SE, Katz SE, Weinberg EO, Lorell BH, Aurigemma GP, Douglas PS. Serial echocardiographic assessment of left ventricular geometry and function in rats with pressure overload hypertrophy: chronic angiotensin-converting enzyme inhibition attenuates the transition to heart failure. Circulation 1995;91:2642–54.
24. Kromer EP, Riegger GAJ. Effects of long-term angiotensin converting enzyme inhibition on myocardial hypertrophy in experimental aortic stenosis in rats. Am J Cardiol 1988;62:161–3.
25. Nichols JR, Gonzalez NG. Increase in myocardial cell cGMP concentration in pressure-induced myocardial hypertrophy. J Mol Cell Cardiol 1982;14:181–3.
26. Sandoff JD, Scholz PM, Tse J, Weiss HR. Increased guanylate cyclase activity is associated with an increase in cyclic guanosine 3′,5′-monophosphate in left ventricular hypertrophy. J Clin Invest 1996;98:838–845.
27. Nava E, Noll G, Luscher TF. Increased activity of constitutive nitric oxide synthase in cardiac endothelium in spontaneous hypertension. Circulation 1995;91:2310–13.
28. Bayraktutan U, Shah AM. Upregulation of type 3 nitric oxide synthase in myocardium of spontaneously hypertensive rats [abstract]. Circulation 1996; 94(suppl):I-521.
29. Hayakawa H, Raij L. The link among nitric oxide synthase activity, endothelial function, and aortic and ventricular hypertrophy in hypertension. Hypertension 1997;29(part 2):235–41.
30. Kolpakov V, Gordon D, Kulik TJ. Nitric-oxide generating compounds inhibit total protein and collagen synthesis in cultured vascular smooth muscle cells. Circ Res 1995;76:305–9.
31. Harding P, Carretero OA, LaPointe MC. Effects of interleukin-1β and nitric oxide on cardiac myocytes. Hypertension 1995;25:421–30.
32. Beckman JS, Koppenol WH. Nitric oxide, superoxide and peroxynitrate: the good, the bad and the ugly. Am J Physiol 1996;271:C1424–C1437.
33. Cernadas MR, Lopez-Farre A, Riesco A, et al. Renal and systemic effects of aminoacids administered separately: comparison between L-arginine and non-nitric oxide donor aminoacids. J Pharmacol Exp Ther 1992;263: 1023–9.
34. Grocott-Mason R, Anning, P, Evans H, Lewis MJ; Shah AM. Modulation of left ventricular relaxation in isolated ejecting heart by endogenous nitric oxide. Am J Physiol 1994;267:H1804–1813.
35. Paulus WJ, Kastner S, Vanderheyden M, Shah AM, Drexler H. Myocardial contractile effects of L-arginine in the human allograft. J Am Coll Cardiol 1997;29:1332–8.
36. Shah AM, Spurgeon HA, Sollot SJ, Talo A, Lakatta EG. 8-bromo-cGMP reduces the myofilament response to $Ca^{2=}$ in intact cardiac myocytes. Circ Res 1994;74:970–8.
37. Gross WL, Bak MI, Ingwall JS, et al. Nitric oxide regulates rat heart contractile reserve and inhibits creatine kinase. Proc Natl Acad Sci USA 1996;93:5604–9.
38. Mery P-F., Lohman SM, Walter U, Fischmeister R. Ca^{2+}-current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. Proc Natl Acad Sci USA 1991;88:1197–1201.
39. Hare, HH. Kim, B., Flavahan, NA, et al. Pertussis toxin-sensitive G proteins influence nitric oxide synthase III activity and protein levels in rat heart. J Clin Invest 1998;101:1424–31.
40. Simmons WW, Closs EI, Cunnigham JM, Smith TW, Kelly RA. Cytokines and insulin induce cationic aminoacid transporter expression in cardiac myocytes: regulation of L-arginine transport and NO production by CAT-1, CAT-2a and CAT-2b. J Biol Chem 1996;271:11694–11702.